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PURIFICATION AND PROPERTIES OF PROTEINASE INHIBITORS FROM *ERYTHRINA CORALLODENDRON* SEEDS

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Key Word Index—*Erythrina corallodendron*; Leguminosae; proteinase inhibitors; inhibitor activities, N-terminal sequence, reactive sites.

Abstract—Four proteinase inhibitors were purified from *Erythrina corallodendron* seeds. Each inhibitor consists of 172–173 amino acids (M_r 20000) including four half-cystine residues, and resembles the Kunitz-type proteinase inhibitors. Inhibitor DE-2 inhibited trypsin strongly and chymotrypsin slightly less effectively. It had no effect upon tissue plasminogen activator (t-PA). Inhibitor DE-3 inhibited trypsin, chymotrypsin and t-PA. Inhibitor DE-5 and DE-10 were relatively specific for α-chymotrypsin, they were poor inhibitors for trypsin and had no effect upon t-PA. The N-terminal amino acid sequence of inhibitor DE-3 resembles those of other *Erythrina* species Inhibitor DE-2 and DE-3 have the same reactive sites (Arg-Ser).

INTRODUCTION

The genus *Erythrina* comprises nearly 110 species of trees, shrubs and herbaceous plants that are widely distributed throughout tropical to warm regions of the world [1]. Many of the species are indigenous to South Africa and the seeds are good sources of proteinase inhibitors. The seeds all contain inhibitors for trypsin and α-chymotrypsin [2] Further, the genus is unusual in that one of the trypsin inhibitors has the unique ability to bind to and inhibit tissue plasminogen activator (t-PA) [3]. The *Erythrina* proteinase inhibitor resembles other inhibitors of the Kunitz-type. They have M_rs of *ca* 20000 and contain about 170 amino acids that are cross-linked by two intramolecular disulphide bonds. The present communication describes the purification and some properties of the proteinase inhibitors from the seeds of *E. corallodendron*. A paper on *E. corrallodendron* seed was published [4] but the seeds are wrongly named and should be called *E. lysistemon*. The *E. corallodendron* seeds are distinguished by a red and black testa and a black hilum-scar [5].

RESULTS

The crude preparation of *E. corallodendron* seeds was fractionated by gel filtration on Sephadex G-50 followed by ion exchange chromatography, first on DEAE-cellulose and then on DEAE-Sepharose as described for other *Erythrina* seeds [6–11]. The purification of the trypsin and chymotrypsin inhibitors is summarized in Table 1. Disc electrophoresis, both in the absence and presence, of dodecyl sulphate showed that proteinase inhibitors DE-2, DE-3, DE-5 and DE-10 each ran as one band This confirmed the homogeneous nature of these inhibitors. Some of the properties of the pure inhibitors are shown in Table 2. Trypsin and chymotrypsin inhibitor DE-3 inhibited t-PA also strongly and has a free *N*-terminal amino acid (valine). Other inhibitors listed in Table 2 have no free *N*-terminal amino acids The *N*-terminal amino acids could be blocked with an acetyl group or a pyroglutamic residue. Table 2 shows that the C-terminal amino acid of inhibitors DE-2, DE-3, DE-5 and DE-10 is serine. In Fig. 1 the *N*-terminal sequence of inhibitor DE-3 from *E. corallodendron* seeds is compared to those of inhibitors from other *Erythrina* seeds. Their sequences of the first 20 amino acids were identical. The amino acid compositions of the pure inhibitors (DE-2, DE-3, DE-5 and DE-10) is given in Table 3.

Treatment of proteinase inhibitors with catalytic amounts of protease at acid pH is known to cause limited hydrolysis at the active site of the inhibitor [12] and thus provides a useful approach to the identification of the reactive sites and their neighbouring sequences. The results of limited hydrolysis of inhibitors DE-2 and DE-3 from the seeds of *E. corallodendron* with trypsin at pH 3 are given in Table 4. SDS-PAGE in the presence of a reducing agent, of the digest, yielded three bands with M_rs of about 20000, 13000 and 7000 representing uncleaved inhibitors (20000) and the products of a single cleavage at the reactive site. For comparison, limited hydrolysis with trypsin of inhibitors DE-3 from *E caffra* and *E. latissima* seeds is included in Table 4 The amino acid sequences immediately C-terminal to the reactive sites of the inhibitors were determined directly on the digest (Table 4). The inhibitor DE-2 (*E. corallodendron*) which contained no free *N*-terminal amino acid, gave one PTH amino acid at each step of the fragment cleaved at the reactive site. In the case of inhibitor DE-3 (*E. corralodendron*) the digest gave two different PTH amino acids for most steps. Since one of each pair could be ascribed to the known *N*-terminal sequence of uncleaved inhibitor, it was possible to assign the other member to the *N*-terminal sequence of the fragment obtained at the reactive site cleavage point.

Table 1 Summary of the purification of proteinase inhibitors from *E. corallodendron* seeds

Steps	Protein (mg)	Total inhibitor activity* (units × 10^3)	Specific inhibitor activity (units/mg protein)	Yield (%)	Bands†
Crude preparation	2000	T 1892	T 946	100	
		C 2244	C 1122	100	
Sephadex G-50	540	T 1485	T 2750	78 5	
		C 1671	C 3094	74 5	
DEAE-cellulose and DEAE-Sepharose					
DE-1	19	T 159	T 8360	8 4	2
		C 61	C 3200	2 7	
DE-2	69	T 582	T 8440	30 8	1
		C 86	C 1240	3 8	
DE-3	7	T 61	T 8720	3 2	1
		C 38	C 5480	1 7	
DE-4	14	T 95	T 6760	5 0	2
		C 90	C 6400	4 0	
DE-5	6	T 1	T 160	0 1	1
		C 51	C 8440	2 3	
DE-6	16	T 7	T 440	0 4	2
		C 123	C 7680	5 5	
DE-7	5	T 2	T 360	0 1	2
		C 35	C 7040	1 6	
DE-8	8	T 0	T 0	0	2
		C 50	C 6320	2.2	
DE-9	27	T 0	T 0	0	2
		C 184	C 6840	8 2	
DE-10	5	T 7	T 1440	0 4	1
		C 23	C 6680	1 5	
DE-11	11	T 5	T 440	0 3	2
		C 71	C 6440	3 2	
DE-12	8	T 55	T 6880	2 9	2
		C 14	C 1720	0 6	

*T, trypsin inhibitor, C, α-chymotrypsin inhibitor
†Disc electrophoresis and SDS gel electrophoresis

Table 2. Some of the properties of proteinase inhibitors from *E. corallodendron* seeds

Property	DE-2	DE-3	DE-5	DE-10
t-Pa inhibitor activities*	1500	60 000	0	0
M_r	20 000	20 000	20 000	20 000
N-Terminal amino acid	None	Val	None	None
C-Terminal amino acid	Ser	Ser	Ser	Ser

*Tissue plasminogen activator inhibitory activity in IU/mg

DISCUSSION

Four inhibitors DE-2, DE-3, DE-5 and DE-10 were isolated and purified from the seeds of *E. corallodendron* by gel filtration followed by ion exchange chromatography. Each inhibitor consists of 172–173 amino acids, cross-linked by two disulphide bridges, has a M_r of *ca* 20 000 and resembles the Kunitz-type proteinase inhibitors. Inhibitor DE-2 inhibited trypsin strongly and chymotrypsin slightly less effectively and it had no effect on tissue plasminogen activator (t-PA). Inhibitor DE-3 inhibited trypsin, chymotrypsin and t-PA. Inhibitors DE-5 and DE-10 were relatively specific for α-chymotrypsin, they were poor inhibitors of trypsin and t-PA. The inhibitors from *E. corallodendron* seeds share many chemical characteristics with Kunitz-type inhibitors from other species of *Erythrina* [2]

The first report of a complete amino acid sequence of a Kunitz-type trypsin inhibitor (from soybeans) was described in 1973 [13–15] Later, the amino acid sequences of two trypsin iso-inhibitors from winged bean seeds [*Psophocarpus tetragonolobus* (L) DC] which resemble

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                                        10                                  20
(a)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(b)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(c)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(d)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(e)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(f)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro-Gln - - -
(g)  Val-Leu-Leu-Asp-Gly-Asn-Gly-Glu-Val-Val-Gln-Asn-Gly-Gly-Thr-Tyr-Tyr-Leu-Leu-Pro - - - -
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Fig 1. Comparison of the *N*-terminal amino acid sequences of Kunitz-type trypsin/chymotrypsin/t-Pa inhibitors from various seeds. (a) *E acanthocarpa* DE-1 [7], (b) *E caffra* DE-3 [8], (c) *E decora* DE-3 [9], (d) *E. humeana* DE-3 [10], (e) *E latissima* DE-3 [6]; (f) *E lysistemon* DE-3 [11] and (g) *E corallodendron* (this paper)

Table 3 Amino acid compositions of proteinase inhibitors from *E corallodendron* seeds*

Amino acid	DE-2	DE-3	DE-5	DE-10
Asp	17 1 (17)	17 2 (17)	20.0 (20)	20.4 (20)
Thr	7.8 (8)	8 6 (9)	9.5 (10)	9 6 (10)
Ser	15 1 (15)	12.3 (12)	12.2 (12)	11.9 (12)
Glu	23.4 (23)	26.2 (26)	24 4 (24)	23.8 (24)
Pro	14.1 (14)	11 9 (12)	11 3 (11)	10 8 (11)
Gly	17.2 (17)	15 5 (16)	14.1 (14)	12.9 (13)
Ala	3 4 (3)	6 6 (7)	10 9 (11)	10 8 (11)
½-Cys	3 3 (4)	3.4 (4)	3 7 (4)	3 7 (4)
Val	12 3 (12)	12.6 (13)	11 8 (12)	12 9 (13)
Met	2 1 (2)	0 3 (0)	1.9 (2)	1.3 (1)
Ile	8 8 (9)	5 7 (6)	6 0 (6)	5 7 (6)
Leu	11.7 (12)	15.5 (16)	15 7 (16)	15.6 (16)
Tyr	8 8 (9)	7 4 (7)	5 2 (5)	5 2 (5)
Phe	6 7 (7)	4 9 (5)	5 3 (5)	5 2 (5)
Lys	8 9 (9)	11 7 (12)	8 6 (9)	9 1 (9)
His	2.3 (2)	2 8 (3)	2 4 (2)	2 8 (3)
Arg	8 0 (8)	7.4 (7)	6 8 (7)	8.3 (8)
Trp	2 0 (2)	1 9 (2)	1 6 (2)	1 7 (2)
	173	172	172	173

*Given as mol of residue per mol inhibitor.

the Kunitz-type inhibitors, were described [16]. Recently, the sequence of the trypsin/chymotrypsin/t-PA inhibitor DE-3 from *E latissima* seeds and the sequence of the trypsin/chymotrypsin/t-PA from *E. caffra* seeds, have been elucidated [17, 18] What is immediately apparent is the high degree of sequence homology between the various inhibitors [18] Further, the inhibitors from the winged bean seeds and the *Erythrina* seeds have the same active site, namely Arg^{64}-Ser^{65}, whereas the inhibitor from soybeans has Arg^{64}-Ile^{65} as reactive site. In spite of this similarity in sequences and also in reactive sites, the inhibitors of winged bean seed and soybeans have no effect on tissue plasminogen activator.

The trypsin inhibitor DE-2 and the trypsin/chymotrypsin/t-PA inhibitor DE-3 from *E corallodendron* seeds have the same reactive sites (Arg-Ser). In addition, the sequences around the reactive sites show a significant degree of sequence homology. It was obvious that trypsin/chymotrypsin/t-PA inhibitor DE-3 from *E. corallodendron* seeds, and trypsin/chymotrypsin/t-PA inhibitor DE-3 seeds of *E latissima* revealed several properties which were identical with regard to the *N*-terminal amino acid sequences, the reactive sites and the sequences around the reactive sites

EXPERIMENTAL

Materials Erythrina corallodendron seeds were collected by SAIDCOR, Pretoria Porcine trypsin (three times crystallized) and bovine α-chymotrypsin were supplied by Miles Labora-

Table 4 Digestion of proteinase inhibitors at the reactive sites with trypsin at pH 3 and the sequence around their reactive sites

Inhibitors		Digestion (%)	Number of bands*	Sequence around the reactive sites
E corallodendron	DE-2	40	3†	–Arg§–Ser–Tyr–Phe–Ile–Pro – – – – –
	DE-3	70	3	–Arg§–Ser–Thr–Phe–Ile–Pro – – – – –
				64 65
E caffra	DE-3	90‡	3	–Arg§–Ser–Ala–Phe–Ile–Pro – – – – –
				64 65
E. latissima	DE-3	90‡	3	–Arg§–Ser–Thr–Phe–Ile–Pro – – – – –

*SDS gel electrophoresis in the presence of a reducing agent.
†M_r 20000, 13000 and 7000.
‡Results taken from ref 2
§Reactive site

tories (Pty) Ltd, Cape Town Recombinant human tissue-type plasminogen activator was obtained from Genentech (San Francisco) DEAE-cellulose was a microgranular preparation (DE-52) from Whatman Sephadex G-50 (fine) and DEAE-Sepharose CL-6B were obtained from Pharmacia

Physicochemical methods Sephadex G-50, DEAE-cellulose, and DEAE-Sepharose columns were prepared as recommended by the manufacturers, and the eluates were monitored at 280 nm with a Beckman spectrophotometer Disc electrophoresis at pH 9 using a 15% gel was performed according to the method of ref [19] SDS gel electrophoresis at pH 7 2 using a 10% gel was carried out a described by Weber and Osborn [20] The bands were stained with Kenacid blue K dissolved in H_2O–HOAc–MeOH (5 1 5).

Proteinase inhibitor assays Trypsin and chymotrypsin inhibitor activities were determined as described [6] Inhibition of tissue plasminogen activator inhibitor was assayed in a plasminogen-independent fluorometric assay as described in ref [3]

Chemical analysis methods. Amino acid analyses were performed on a Waters HPLC using the *o*-phthalaldehyde fluorescence detection system [21] Samples were hydrolysed with 6 M HCl for 24 hr in sealed evacuated tubes, PhOH was added to prevent destruction of tyrosine [22] Half-cystine was determined as cysteic acid by the method of ref [23] For the determination of tryptophan, the samples were hydrolysed with 3 M toluene-*p*-sulphonic acid as described in ref [24]

Limited hydrolysis with trypsin The procedure used for the limited hydrolysis at pH 3 of the inhibitors by trypsin was similar to that described in ref [25] The digestions were carried out for 7 days at 20°

Sequence determinations The *N*-terminal sequences of the uncleaved inhibitor and the fragments of inhibitors obtained by limited hydrolysis, at pH 3, with trypsin were determined with a spinning cup sequencer (Beckman) converted to a gas phase instrument as described in ref [26] The programme used was similar to that described in ref [27] The phenylthiohydantoin (PTH) derivatives of amino acids were identified with HPLC using a micro-Bondapak C_{18} column (Waters Associates Inc)

Carboxy-terminal analysis The *C*-terminal amino acids of the proteinase inhibitors and the fragments were determined by the hydrazinolysis method as described in ref [28]

Crude proteinase inhibitors The preparation of the crude proteinase inhibitors was as described in ref [6] The yield was 18 0 g from 100 g *E. corallodendron* seeds

REFERENCES

1 Barneby, K C, Krukoff, B A and Raven, P-H (1982) *Erythrina Symposium* IV *Erythrina* (*Fabaceae· Faboideae*) Allertonia Pacific Tropical Botanical Garden, Lawai, Kauai, Hawaii, **3**, 154

2 Joubert, F J, Merrifield, E H and Dowdle, E B D (1987) *Int J Biochem* **19**, 601

3 Heussen, C, Joubert, F and Dowdle, E B (1984) *J Biol Chem* **259**, 11635

4 Joubert, F J and Sharon, N (1985) *Phytochemistry* **24**, 1169

5 Hennessy, E F (1972) *South African Erythrinas* The Natal Branch of the Wildlife Protection and Conservation Society of South Africa Colorgraphic, Durban

6 Joubert, F J, Carlsson, F H H and Haylett, T (1981) *Hoppe Seyler's Z Physiol Chem* **362**, 531

7 Joubert, F J (1982) *J Natl Prod* (*Lloydia*) **45**, 427

8 Joubert, F J (1982) *Int J Biochem* **14**, 187

9 Joubert, F J (1986) *S Afr J Chem* **39**, 169

10 Joubert, F J (1982) *S Afr J Chem* **35**, 67

11 Joubert, F J (1982) *Phytochemistry* **21**, 1213

12 Ozaka, K and Laskowski, M Jr (1966) *J Biol Chem* **241**, 3955

13 Koide, T and Ikenaka, T (1973) *Eur J Biochem* **32**, 401

14 Koide, T and Ikenaka, T (1973) *Eur J Biochem* **32**, 417.

15 Koide, T, Tsunasawa, S and Ikenaka, T (1973) *Eur J Biochem* **32**, 408.

16 Yamamoto, M, Hara, S and Ikenaka, T (1983) *J Biochem* (*Tokyo*) **94**, 849

17 Joubert, F J, Heussen, C and Dowdle, E B D (1984) *J Biol Chem* **260**, 12948

18 Joubert, F J and Dowdle, E B D (1987) *Thrombosis Haemostasis* **57**, 356

19 Ornstein, L and Davis, B J. (1962) *Disc Electrophoresis* Reprinted by Distillation Products Industries, Rochester, New York

20 Weber, K and Osborn, K (1969) *J Biol Chem* **244**, 4466

21 Waters Associates Inc Model 420 fluorescence detector instrument manual No IM 82902 July 1980 Rev. C

22 Sanger, F and Thompson E O P (1963) *Biochim Biophys Acta* **71**, 468.

23 Hirs, C H W (1967) *Meth Enzym* **11**, 59

24 Liu, T Y and Chang, Y H (1971) *J Biol Chem* **246**, 2842

25 Birk, Y, Gertler, A and Khalef, S (1967) *Biochim Biophys Acta* **147**, 402

26 Brandt, W F., Aik, H, Chauhan, M and von Holt, C (1984) *FEBS Letters* **174**, 228

27 Hewick, R M, Hunkapiller, M W, Hood, L E and Dreyer, W J (1981) *J Biol Chem* **256**, 7990

28 Narita, K, Matsuo, H and Nakajima, T (1975) in *Protein Sequence Determination, Molecular Biology, Biochemistry and Biophysics* Vol 8, 2nd Edn (Needleman, S B ed) pp 30–143 Springer, Berlin